

CLONAL VARIATIONS IN *PSEUDOMONAS AERUGINOSA*

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1. INTRODUCTION

The genetic diversity within a bacterial species is determined by the number and size of chromosomal and extrachromosomal elements, rates of nucleotide substitution, recombination, genome rearrangements and gene flow, and both the size and growth of the bacterial population. Most species of bacteria that were initially analyzed, were of a clonal nature.⁷⁷ The structural characteristics of a clonal population are the paucity of genotypes, linkage disequilibrium among gene loci, and recovery of closely related genotypes over large geographic areas and/or over long periods of time. The accumulation of molecular data during the last 15 years and the growing evidence of the occurrence of horizontal gene transfer among bacteria in nature, however, have led to consideration that bacterial populations are not invariably clonal but range from the highly sexual *Neisseria gonorrhoeae* to the almost strictly clonal *Salmonella*.⁸⁰

The metabolically versatile *Pseudomonas aeruginosa* is present in soil and aquatic habitats, but it is also an important opportunistic pathogen for humans, animals, and plants. Typing of strain collections in single nucleotide polymorphisms (SNPs), DNA fragment length polymorphisms and phenotypic traits indicated that the current *P. aeruginosa* population is in linkage equilibrium and consists of a net of equivalent genotypes (termed clones), whereby a subset of clones is overrepresented due to epidemic spread.^{36,60} Isolates from the

inanimate environment and clinical habitats have been shown to share the same chemotaxonomic profile²³ and repertoire of metabolic and virulence traits.¹ Irrespective of their origin, isolates from disease and environment were similarly proficient in the degradation of environmental pollutants and secretion of virulence factors.¹ In other words, there are no disease- or habitat-associated clones. However, we do observe adaptation of *P. aeruginosa* to a particular niche. Most data exists of how *P. aeruginosa* colonizes and persists in the atypical habitat of the cystic fibrosis (CF) lung where independent of the genetic background of the clone a convergent evolution towards common phenotypes takes place.⁸⁹

This chapter summarizes our current knowledge about the inter- and intracolon diversity of genotype and phenotype of *P. aeruginosa*.

2. INTRA- AND INTERCLONAL GENOME DIVERSITY

Physical mapping and sequencing and Southern hybridization data indicate that the *P. aeruginosa* genome is made up of a mosaic of a conserved core and variable accessory segments.^{20,31,36,66,84} The core genome is characterized by a conserved synteny of genes and a low average nucleotide substitution rate. Clone- or strain-specific genome islands and genome islets define the accessory part of the chromosome and lead to fluctuations in the genome size, which can range from 5.2 to 7 Mbp.⁷³

2.1. Clonal Variation of the Core Genome

The complete genome sequence of strain PAO1⁸⁵ is the genetic blueprint for *P. aeruginosa*. Genomic DNA hybridization of in total 39 *P. aeruginosa* strains of diverse origin onto PAO1 microarrays^{20,95} detected the presence of almost 90% of the 5570 predicted PAO1 protein coding sequences in all strains. Hence, the core genome is made up of about 5000 highly conserved genes.

Interclonal sequence variation is low in the *P. aeruginosa* core genome. Comparative sequencing of housekeeping genes in strain collections revealed an average rate of sequence polymorphism of 0.3%, which is about one order of magnitude lower than in comparable housekeeping genes of *Salmonella enterica*.³⁶ The ratio of non-synonymous to synonymous nucleotide substitutions is about 1:6. Sequence variation within clones is substantially lower than the already low sequence diversity amongst unrelated clones: Within 300 kb of bulk sequence, just a single synonymous nucleotide substitution was detected in one of four analyzed strains.^{36,43} In other words, members of a clone are characterized by virtually identical core genome sequence in all segments with low sequence diversity.

Figure 1 shows the comparison of 49 single nucleotide substitutions (SNPs) of *P. aeruginosa* detected in *oriC*, *ampC*, *citS*, *fliC*, *oprI* with 500 SNPs of *S. enterica* detected in *gapA*, *putP*, and *mdh*.³⁷ In contrast to the high GC

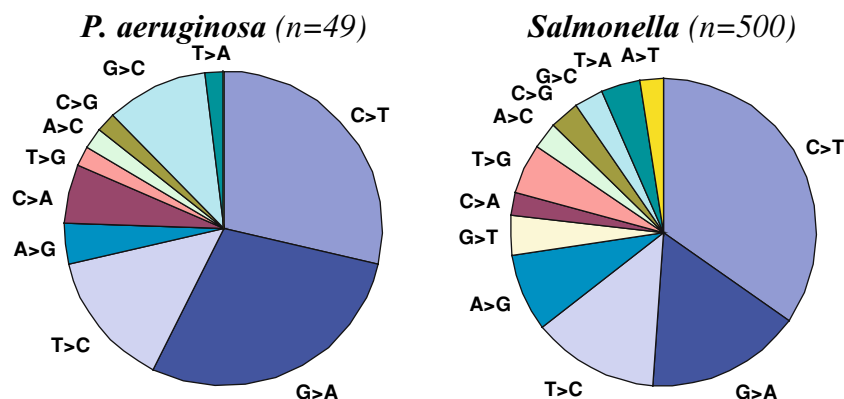


Figure 1. Pie charts showing all single base substitutions detected in *oriC*, *citS*, *ampC*, *oprI*, and *fliC* sequences of 18 *P. aeruginosa* strains and in *gapA*, *putP*, and *mdh* of 16 *Salmonella* strains.

content of the bulk *P. aeruginosa* chromosome (67%), the phylogenetically closely related but ecologically distinct *S. enterica* exhibits a much lower GC content (50–53% GC) and a less pronounced codon usage bias.⁹³ The quantitative distribution of nucleotide substitution types is similar in both bacterial species except for the more frequent G→C transversion in the GC rich *P. aeruginosa*. In spite of their dissimilar GC content and codon usage bias, about 75% of nucleotide substitutions are transitions. C→T is the most abundant substitution, followed by G→A, T→C, and A→G (Figure 1). Both nucleotide substitution profile and transition-to-transversion ratio are non-randomly distributed. The observed bias probably reflects first, the thermodynamic stability and geometric selection of the mismatches; and second, evolutionarily conserved error-correction mechanisms, i.e. the preferential repair of lesions on the transcribed strand.³⁷ This argument that nucleotide sequence variation is not governed by chromosomal GC content and codon usage but by DNA structure and repair is substantiated by the finding that the same profiles seen in bacteria were also observed for sequence variants and disease-causing mutations in phylogenetically distant mammalian genomes.³⁷

Twenty-five regions of significantly elevated sequence variation were uncovered in pairwise comparisons between PAO1 and three other partially sequenced strains (Table 1).⁸⁴ There were no obvious sequence features unique to these regions that flag them relative to the rest of the genomic sequence, albeit a lower average GC contents and an underutilization of the most frequently used codons were noted.

The genome segments with the highest level of sequence diversity are the genes whose products are involved in flagellar biosynthesis and genes whose products are involved in the biosynthesis of the siderophore pyoverdine and the receptor for ferripyoverdines.⁸⁴ Pyoverdine is the primary siderophore of

Table 1. PAO1 sequence coordinates for regions of high sequence diversity^a (modified from Table 3 in ref [84] by permission of the authors and the American Society for Microbiology).

Coordinates ^b of PAO1		Length PAO1 (bp) ^c	No. of SNPs ^d	No. of bases available ^e	%SNPs ^f	PAO1 ORFs ^g
Start	End					
290,000	294,999	5000	83	1488	5.58	PA0259–PA0262
320,000	324,999	5000	97	1754	5.53	PA0285–PA0289
515,000	519,999	5000	143	2103	6.80	PA0457–PA0459
530,000	534,999	5000	111	2288	4.85	PA0470–PA0473
555,000	559,999	5000	66	1252	5.27	PA0495–PA0500
652,500	657,499	5000	76	1171	6.49	PA0594–PA0596
685,000	689,999	5000	44	789	5.58	PA0625–PA0633
695,000	699,999	5000	84	1436	5.85	PA0640–PA0643
790,000	799,999	10,000	274	2535	10.81	PA0719–PA0731
1,060,000	1,064,999	5000	57	926	6.16	PA0976–PA0982
1,170,000	1,174,999	5000	224	1881	11.91	PA1084–PA1087 ^h
2,150,000	2,154,999	5000	88	842	10.45	PA1967–PA1972
2,550,000	2,554,999	5000	59	1346	4.38	PA2312–PA2317
2,635,000	2,654,999	20,000	451	5787	7.79	PA2383–PA2397 ⁱ
2,660,000	2,664,999	5000	115	551	20.87	PA2399 ⁱ
2,672,500	2,677,499	5000	186	1132	16.43	PA2402 ⁱ
2,682,500	2,692,499	10,000	139	1723	8.07	PA2402–PA2409 ⁱ
3,647,500	3,652,499	5000	81	1417	5.72	PA3260–PA3264
4,060,000	4,064,999	5000	85	1695	5.01	PA3624–PA3629
5,037,500	5,042,499	5000	98	1923	5.10	PA4500–PA4503
5,070,000	5,074,999	5000	162	2030	7.98	PA4526–PA4532
5,097,500	5,102,499	5000	119	1825	6.52	PA4549–PA4554 ^j
5,187,500	5,192,499	5000	91	1561	5.83	PA4625
5,722,500	5,727,499	5000	162	1966	8.24	PA5084–PA5089
6,090,000	6,094,999	5000	93	618	15.05	PA5412–PA5415

^a Sequencing data for all three strains that had been subjected to whole genome shot-gun sequencing was compared to the PAO1 reference in 5000-bp sliding windows that were sequentially offset by 2500 bp. Regions with at least 500 bp of alignable sequence that exhibited nucleotide diversity values greater than three standard deviations (>4.35% sequence differences) from the mean value of 0.5% are shown.

^b Sequence coordinates of the PAO1 reference sequence, accessible at www.pseudomonas.com.

^c Overall span of the PAO1 region encompassed by high sequence variation.

^d Number of SNPs detected.

^e Total number of alignable bases in which SNPs were detected.

^f Percent SNPs among alignable bases.

^g Annotated ORFs within high-diversity regions. A more comprehensive description of these genes is available at www.genome.washington.edu/UWGC and at www.pseudomonas.com.

^h Flagellar biogenesis genes.

ⁱ Pyoverdine locus.

^j Minor type IV pili prepilin.

P. aeruginosa. Each strain makes one of three pyoverdine types, each type with a distinct peptide chain that is synthesized non-ribosomally. The pyoverdine region spans an interval of approximately 50 kb in PAO1. The three divergent sequence types correspond to the three structural types of pyoverdines.⁷⁹ The outer-membrane pyoverdine receptor, FvpA (PA2398), is also type-specific, transporting only its corresponding pyoverdine. FvpA exhibits the largest variations with about 50% mismatch of amino acid pairwise alignment between genes of each pyoverdine type.^{15,42,63} FvpA moreover shows substantial intratype variation and apparently accumulated non-synonymous changes at an elevated rate which has been interpreted as strong evidence of positive selection.^{25,79} The next most divergent genes with 15–40% mismatch of amino acid pairwise alignment are immediately adjacent to *fvpA*, and include the ABC transporter *pvdE* (PA2397) and the non-ribosomal peptide synthetase genes *pvdD*, *pvdJ*, and *pvdI* (PA2399–PA2402). Besides *fvpA* 10 further hotspots of elevated intratype sequence divergence were identified. Since these islands of about 100 bp in length are located within regions that are divergent between pyoverdine types and since intratype differences are very similar to those between pyoverdine types, the sequence divergence probably arose from recombinations.⁷⁹

The flagellin biosynthesis genes encode the elements for the serologically distinct a- and b-type flagellae. The flagellum confers motility and chemotaxis, facilitates adherence to cells and inanimate surfaces and contributes to the colonization and invasion of hosts during infection. Flagellins, a- and b-type, are 74% identical in the nucleotide sequence and 63–65% identical in the amino acid sequence.^{7,82,94} They share nearly identical N- and C-terminal sequences, whereas the central region is variable in size and primary structure. This central part is also the major region of intratype sequence variation among a-type *fliC* genes. Based on the amino acid sequences of flagellins from 24 a-type *P. aeruginosa* strains, two subtypes, A1 and A2 were recognized that differ in the central regions by 13 amino acid substitutions and two small deletions of three- and four- amino acids.⁷ Although a-type and b-type flagellins differ by 37–38% in their primary structure, the impact of sequence diversity on secondary and tertiary structure is low. A1, A2, and b-type flagellins match perfectly in their profiles for hydrophobicity, flexibility of the peptide backbone, antigenic index, and probability of surface exposure.⁸³ The constraints for the efficient multimerization of subunits to a functional flagellum are probably so tight that the polymorphic proteins fold into a similar three-dimensional structure. a-type flagellins are glycosylated.¹² a-type strains carry a polymorphic genomic island that is essential for glycosylation of flagellin.⁴ An a-type strain either harbors the long version of the island of 14 open reading frames (*orfA* to *orfN*) or an abbreviated version (short island) in which *orfD*, *-E*, and *-H* are polymorphic and *orfI*, *-J*, *-K*, *-L*, and *-M* are absent.⁷ The glycosylation island is located upstream of *fliC*. Comparative sequencing between strains PAK and PAO1 as representatives for a- and b-type flagella revealed that the polymorphic region

of the flagellar regulon encompasses the region from *flgK* (PA1086) at the 5' end up to amino acid 88 of *fleP* (PA1096) at the 3' end: 5'-*flgKL* – glycosylation island – *fliC* – *fleL* – *fliDSS'* – *fleP*.^{4,84} Correspondingly, there are two types of flagellar cap proteins, FliD, which are only 58% identical at the nucleotide level and 43% identical at the amino acid level.⁵ These genes are co-inherited with their cognate flagellin gene types, a or b.

The most substantial interclonal sequence variation for a single gene common to all *P. aeruginosa* is observed for the *pilA* gene (PA4525) encoding the type IV pili that play a major role in mediating the adhesion of the bacterial cell to host tissue. All classic pilin subunits share characteristic features, including a six- or seven-amino acid leader peptide, an *N*-methylated phenylalanine as the first residue of the mature protein and a highly conserved N-terminus with 25–30 hydrophobic amino acids, but otherwise the primary sequence is highly variable. The published *pilA* sequences segregate into five groups exhibiting less than 30% nucleotide identity that provide fewer homologies between themselves than with pilins of different species.^{36,81} Each group carries a specific sequence insertion downstream of *pilA*. Group I members share about 85%, group II members about 65% nucleotide identity amongst themselves.¹³ The type IV pili of *P. aeruginosa* are no more closely related to each other or to other γ -Proteobacteria genera *Escherichia*, *Aeromonas*, *Vibrio*, and *Moraxella* than they are to the pili of the β -Proteobacteria *Neisseria* and *Eikenella*, the genus *Dichelobacter*, representative of the deepest branching γ -Proteobacteria, or the phylogenetically distant δ -Proteobacterium *Myxococcus*.⁸¹ *P. aeruginosa* probably acquired its pilin genes from the *Moraxella* lineage, because the *pilA* genes still retain the GC and codon usage characteristics of *Moraxella* pilin genes.⁴⁸

The type III secretion system as one of the major virulence determinants of *P. aeruginosa* transports four known effector proteins: ExoS, ExoT, ExoU, and ExoY. The bifunctional ExoS exerts its cytotoxic activities by a GTPase-activating domain and a ADP-ribosyltransferase activity.⁸ ExoT is also an ADP-ribosyltransferase but has only 0.2% of the catalytic activity of ExoS. Like ExoS, it is a GTPase-activating protein for Rho GTPases. ExoU is a potent patatin-like phospholipase that causes rapid cell death following its injection into host cells.⁷¹ ExoY is an adenylate cyclase that elevates the intracellular cAMP levels in eukaryotic cells and causes rounding of certain cell types.⁹⁰ The genes encoding the secretion, translocation, and regulatory machinery of the type III secretion system are clustered together in the *P. aeruginosa* chromosome. The genes encoding the type III effector proteins, however, are scattered throughout the chromosome. In an epidemiological study on 115 *P. aeruginosa* isolates²² the large chromosomal locus and *exoT* (PA0044) were present in all isolates. In contrast, the *exoS* (PA3841), *exoU*, and *exoY* (PA2191) genes were variable traits. Overall, 72% of examined isolates contained the *exoS* gene, 28% contained the *exoU* gene, and 89% contained the *exoY* gene. An inverse correlation was noted between the presence of the *exoS* and *exoU* genes in that all isolates except two, one containing both genes and another containing neither

of them, contained either *exoS* or *exoU* but not both. No significant difference in *exoS*, *exoU*, or *exoY* prevalence was observed between clinical and environmental isolates or between isolates cultured from different disease sites except for respiratory isolates from patients with CF. CF isolates harbored the *exoU* gene less frequently and the *exoS* gene more frequently than did isolates from some of the other sites of infection, including the respiratory tract of patients without CF. These results suggest that the *P. aeruginosa* type III secretion system is present in nearly all clinical and environmental isolates but that individual isolates differ in their effector genotypes.

P. aeruginosa lipopolysaccharide (LPS) is composed of lipid A, the core oligosaccharide, and the long chain polysaccharides (O-antigen) (detailed information in the article by Lam *et al.*, volume 3, Chapter 1 of this monograph series). In short, the majority of *P. aeruginosa* produces two distinct forms of O-antigens called A-band and B-band. Differences in the chemical structure of the B-band LPS are responsible for the serogroup specificity of the respective strains and has been employed for many years for serotyping of *P. aeruginosa* isolates. The major set of enzymes responsible for O-antigen B-band synthesis and assembly are encoded in a single, large gene cluster (PA3160–PA3141 in strain PAO1). Raymond *et al.*⁶⁴ sequenced this B-band gene island in all 20 IATS reference serotype strains. Eleven groups of gene clusters were identified that are highly divergent from one another at the DNA sequence level. Within each group a high degree of sequence conservation was observed. The B-band gene islands of serotypes O1, O4, O6, O9, and O12 constitute each a distinct gene cluster. Groups with two members include O3 and O15 (Lory), O7 and O8, O10 and O19, O11 and O17, and O13 and O14. The largest group with 98% sequence identity contains strains of serotypes O2, O5, O16, O18, and O20, consequently the variations in the structures among these serotypes are not conferred by the B-band gene island. This argument also applies to the O10–O19 group, for which no DNA sequence differences were found in 16 kbp of sequence, and to the O7–O8 group, in which only two conservative amino acid changes were identified.

In summary, to date the following genes and gene clusters exhibit the largest interclonal genetic diversity in the core genome: the pyoverdine locus, the flagellar regulon, *pilA*, the type III secretion effector proteins and the O-antigen biosynthesis locus. Each locus is present in all strains, but the genes in each locus are highly divergent between strains. This “replacement island” phenomenon presumably results from diversifying selection, a type of selection that maintains multiple alleles in the population.⁷⁹

Mosaic genes are a further source of genetic diversity. Evidence for a mosaic gene structure is drawn from SNP haplotype^{36,83} or the detection of cassettes.⁸² Our current knowledge about mosaic genes in *P. aeruginosa* is restricted to *ampC* (PA4110),⁸³ *fleP* (PA1096),⁴ *fliC* (PA1092),^{36,83} *mucABCD* (PA0763–PA0766),¹¹ and *oprD* (PA0958).⁶¹ The *ampC* sequences of 18 strains were compiled into 12 groups by their diagnostic SNP patterns.⁸³ No

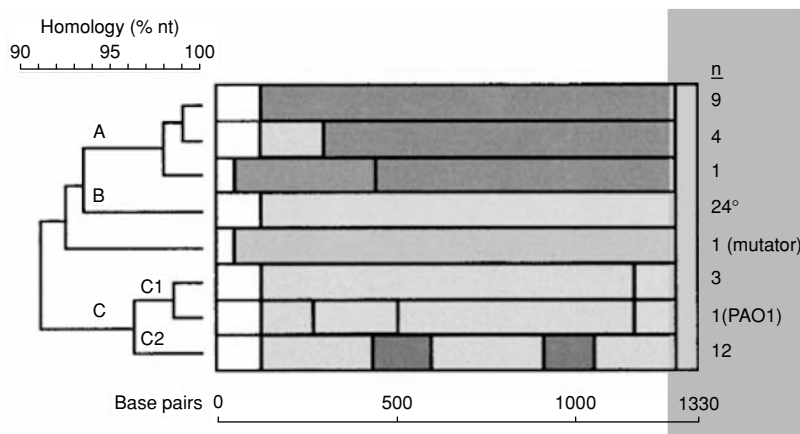


Figure 2. Graphical representation of the mosaic structure of the *oprD* gene in 55 unbiased *P. aeruginosa* strains. Including 10 clone C isolates; *n*, number of isolates belonging to a subgroup. Reproduced from the article by Pirnay *et al.*⁶¹ by permission of the authors and Blackwell Publishing.

linkage of dimorphisms was observed which indicated repetitive intragenic recombination events. However, the low nucleotide substitution rate and the low number of analyzed strains did not allow statistical evidence of a putative mosaic structure of *ampC*. *fleP* is a mosaic gene because the 3' end of the polymorphic region of the flagellar regulon is located within *fleP*.⁴ The first 264 nucleotide of *fleP* were only 56% identical between strains PAK and PAO1 but the last 31 nucleotides of *fleP* were 100% identical. a-Type *fliC* genes contain a variable 141-bp central cassette showing 28% nucleotide and 40% amino acid diversity.⁸² Significant non-random clustering of polymorphic sites within this cassette indicated an intragenic recombination event and a mosaic gene structure.³⁶ Sequencing of 37 CF *P. aeruginosa* isolates in the *mucABD* operon uncovered 16 SNP genotypes. The non-random distribution of conserved SNP blocks visualized the mosaic structure of the *muc* operon.¹¹ Sequence analysis of *oprD* in 55 *P. aeruginosa* isolates, collected over a period of 15 years from various, spatially separated, clinical and environmental habitats, uncovered a microscale mosaic structure of *oprD*.⁶¹ All sequences fell into three main groups, which differ by 7–9% of nucleotides. Several recombinational exchanges of DNA blocks of 100–300 bp led to a mosaic gene structure and caused a further divergence into subgroups (Figure 2).

Our knowledge about sequence variation resides on the complete genome sequence of two strains, whole genome shot-gun sequencing in another three strains and comparative sequencing of strain collections in 11 loci. Considering this rather limited body of comparative sequence data the proportion of five genes with intragenic mosaicism is substantial. In other words, intragenic recombination may be a major driving force for genetic diversity of *P. aeruginosa*.

2.2. Clonal Variation of the Accessory Genome

Genome diversity is accomplished by sequence variation in coding and non-coding regions of the core genome and by a differential repertoire of the accessory genome the latter being made up of genome islands and genome islets and of mobile genetic elements such as phages, plasmids, and transposons. The reader is referred to volume 1, Chapters 6–8 to get comprehensive information about the features of phages, plasmids, and transposons in *Pseudomonas*. This chapter focuses on the variation of chromosomal contents.

Diversity of the *P. aeruginosa* chromosome was first studied by Southern hybridization analysis.^{31,66} *SpeI* macrorestriction fragment length diversity was scanned in 60 unrelated clones for using probes of known map position of the PAO1 chromosome. The *oriC*-containing *SpeI* fragment was the most conserved *SpeI* fragment on the chromosome. Small insertions or deletions lead to a variation of $\pm 10\%$ of chromosomal contents in this region of the origin of replication (Figure 3). Few fragment length classes were seen for most analyzed segments indicating an intermediate range of diversity. In contrast, extensive genomic diversity was detected around the *pilA* and *lipH* loci that later turned out to be hotspots for the integration of genome islands (see below). In other words, the gene contig of the core genome is interrupted by few islets around *oriC* as one extreme and by large segments around *pilA* and *lipA*. Heuer *et al.*³¹ studied the same strain collection by probing the chromosome in four regions with 40–114 kb large PAO1 *SpeI* fragments cloned into yeast artificial chromosomes (YACs). In one region the broad distribution of hybridizing *SpeI* fragment size indicated substantial genome plasticity, but otherwise only few bands within narrow fragment length classes reacted with the probe. The low complexity of the hybridization pattern indicates that conserved PAO1 coding and non-coding sequence is maintained as contigs in *P. aeruginosa*. Intrachromosomal shuffling of sequence is rare. In other words, gene order established for strain PAO1 should be valid for most *P. aeruginosa*. YAC hybridizations compare genomes at low resolution so that the disruption of the sequence contig by small genome islets is not resolved. Indirect evidence for the presence of such small insertions and deletions was provided by the strain-to-strain variation of up to 10% in macrorestriction fragment size.

Information about the PAO1 accessory genome in terms of genome islets and genome islands has meanwhile been obtained by hybridization of genomic DNA from strain collections onto PAO1 microarrays. Wolfgang *et al.*⁹⁵ analyzed 18 strains of diverse origin. Strain-specific genes were localized to 90 discrete regions relative to the PAO1 genome. Many of these regions are composed of small gene blocks (one to four genes) that showed variability in one or more strains. These variable blocks likely contain genes that are highly polymorphic at the nucleotide sequence level or are gained or lost through local recombination events. A second pattern, which was more readily apparent, is characterized by large clusters of tandem genes that show varying levels of

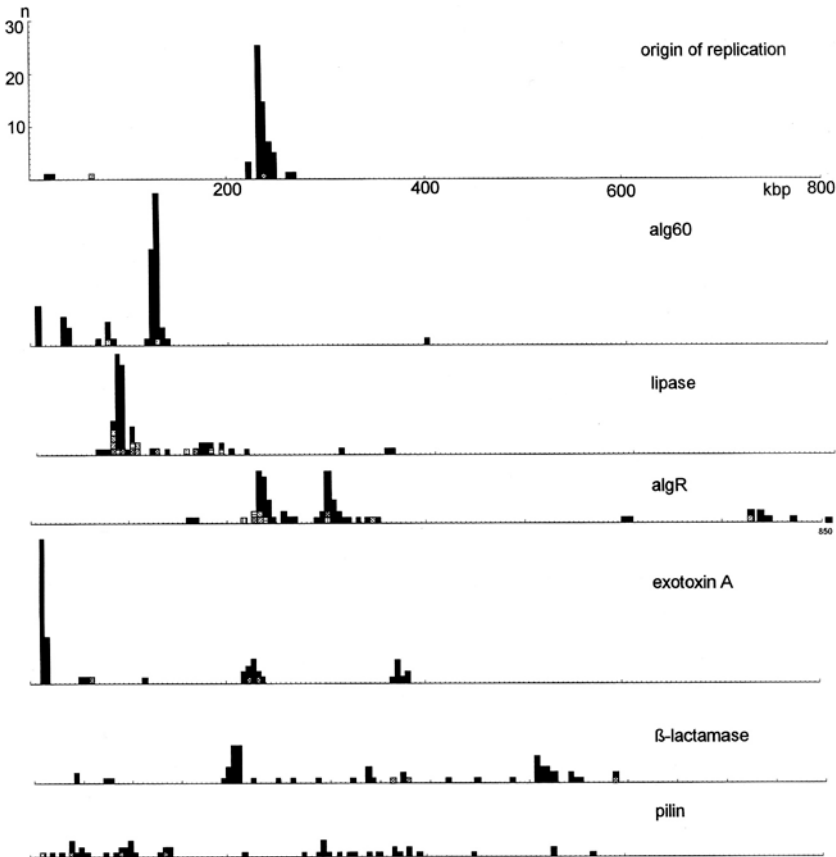


Figure 3. Southern analysis of *P. aeruginosa* clones: size variation of hybridizing *SpeI* fragments. The number of fragments detected by one probe is given as a function of size. Fragment length was counted in 5 kbp increments. Fragment size variation within a clone is indicated by identical open symbols.

polymorphism between strains. Twenty-four of these regions (termed variable segments) were identified. These variable segments are scattered throughout the genome; however, nine segments are immediately adjacent to tRNA or tmRNA genes. Ernst *et al.*²⁰ detected 38 PAO1 gene islands to be absent or divergent in at least 2 out of 14 examined clones. Besides the pyoverdine cluster, the flagellar regulon and the O-antigen biosynthetic gene cluster described above, a further not yet characterized exopolysaccharide is encoded by a gene island (PA1383–PA1393). Numerous islands in *P. aeruginosa* encode pyocins or phage proteins.²⁰ Six further islands were each adjacent to 1 of the 10 members of the *vgr* gene family, genes associated with rearrangement hotspots in the *E. coli*

chromosome. Seven of these 38 islands belong to the subset of 10 chromosomal regions whose low G + C content suggested that they were sites of recent horizontal transfer in PAO1.^{85,95}

The hotspots for gene island replacement are apparently the regions where most intra- and interclonal genome diversity takes place. Intracolonial genome diversity has so far been studied in 21 *P. aeruginosa* isolates of clone C.⁶⁷ Clone C is one of the major clones in the *P. aeruginosa* population and has frequently been isolated from inanimate and disease habitats.^{18,68} Clone C consists of closely related genotypes (also called clonal variants), each of which is characterized by a unique macrorestriction fragment pattern. Within clone C the total genome size varies at maximum by 300 kb. In total 34 different insertions or deletions were mapped that each were present in 1 to 13 strains. The acquisition and loss of DNA occurred preferentially around the terminus of replication but was not observed around the origin of replication, from about *rrnC* to *rrnA* (Figure 4). Three regions close to the *phnAB*, *pilA*, and *lipH* loci were subject to extensive variation processes. These hypervariable regions of the clone C chromosomes match with the hotspots of variation in the Southern and PAO1 microarray hybridization experiments. Subsequent sequencing revealed that most larger genome islands are located in these regions.

The ca. 110 kb large hypervariable region located near the *lipH* gene was sequenced in two clone C strains, strain C and strain SG17M.⁴³ In both strains the region consists of an individual strain-specific genome island of 111 (strain C) or 106 (SG17M) open reading frames (ORFs) and of a 7 kb stretch of clone C-specific sequence of nine ORFs. The left boundary of the islands is a cluster of tRNA genes comprising one tRNA^{Glu} gene followed by two identical tRNA^{Gly} genes separated by 84 bp, one serving as the integration site for the *P. aeruginosa* genome island PAGI-2 in strain C, the other for PAGI-3 in SG17M. PAGI-2 and PAGI-3 terminate at the right end with the terminal 16 and 24 nucleotides of the 3' end of the tRNA^{Gly} gene, respectively. The same organization is seen for the *Pseudomonas clc* genome island that contains the genes encoding the degradation of 3-chlorobenzoate (see Chapter 16 by J.R. van der Meer in this volume for more information). In all three islands the first ORF adjacent to the tRNA^{Gly} gene encodes a bacteriophage P4-related multidomain integrase with an unusual transposase-like C-terminus. PAGI-2 and PAGI-3 have a bipartite structure. The first part adjacent to the tRNA gene consists of strain-specific ORFs encoding metabolic functions and transporters, the majority of which has homologs of known function in other eubacteria. The second part is made up of a syntenic set of ORFs the majority of which is classified as conserved hypotheticals. Forty-seven of these ORFs are arranged in the same order in both islands with a pairwise amino acid identity of 35–88% (Figure 5). Interestingly, PAGI-2 is also found with 100% sequence identity in the *Ralstonia metallidurans* CH34 chromosome⁴³ indicating that first, the genome island is also present in other phylogenetically distant taxa,

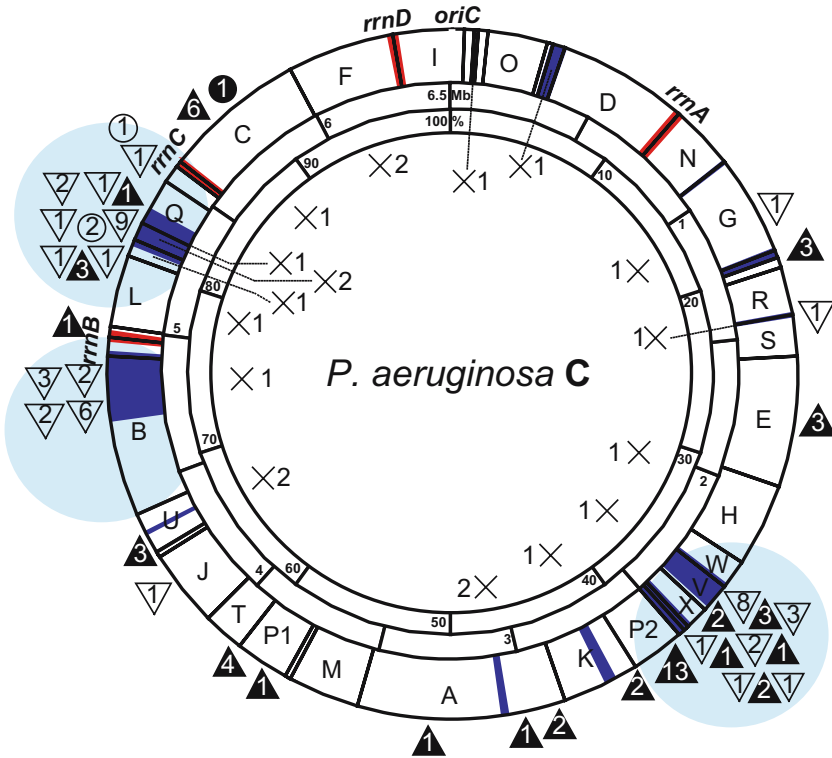


Figure 4. *SpeI* restriction map of *P. aeruginosa* C summarizing all chromosomal changes that occurred within the 21 analyzed clone C strains. Open triangles represent deletions, filled triangles represent insertions, open circles indicate deletion of a *SpeI* site, and filled circles indicate additional *SpeI* sites. Crosses indicate endpoints of recombination. Numbers in the symbols refer to the frequency of an additional genome alteration. Shaded regions indicate additional genetic material in strain C in comparison to PAO1. The hotspots of gene replacement around the *pilA* and *lipH* loci are indicated by the large circles.

and second, this type of island may have closer homologs in other clones and taxa than within the same clone. Subsequent hybridization analyses revealed that additional copies of PAGI-2 that first had been sequenced in a *P. aeruginosa* isolate from a German CF patient's lung, were present in the majority of tested *R. metallidurans* and *R. campiniensis* isolates from wastewater and polluted habitats in Europe and North America.³⁸

PAGI-2 and PAGI-3 are prototypes for tRNA-associated gene islands that are causative for the genetic make-up of one of the hypervariable areas of the *P. aeruginosa* chromosome. The other two hypervariable regions in the *P. aeruginosa* chromosome with pronounced genomic variability reside in the

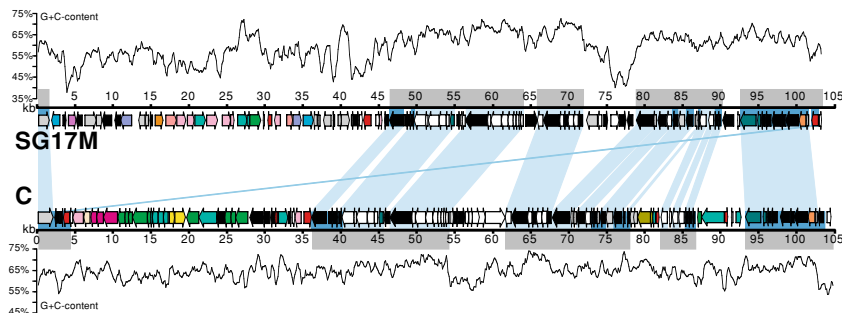


Figure 5. Comparison of the strain-specific gene islands in the *P. aeruginosa* clone C strains SG17M (upper line) and C (lower line). Gene are represented by arrows. Homologous ORFs are linked by light bars. Genes with homologs in the *Xylella fastidiosa* genome island⁷⁸ are highlighted with a dark background. Gray boxes above and below the gene maps mark the syntenic set of core genes that are characteristic for this type of island.⁵¹ Reproduced from the article by Larbig *et al.*⁴³ with permission by the authors and the American Society for Microbiology.

vicinity to the *pilA* and *oprL* – *phnAB* loci. The duplicated copies of a tRNA^{Lys} gene were identified as the hotspots for the integration and excision of DNA in these regions. The large plasmid pCLK106 sequentially recombined with either of the two tRNA^{Lys} genes in *P. aeruginosa* clone K strains, giving rise to reversible rearrangement of a 106 kb genome island in sequential isolates from CF patients³⁵ (Figure 9). In all investigated clone K strains, both episomal and chromosomal copies were detected. During the propagation of single colonies on agar plates *in vitro*, progeny that had retargeted pCLK106 into the other tRNA^{Lys} locus were regularly observed, indicating that pCLK106 is mobilized and reintegrated into the clone K chromosomes at high frequency.

In strain PAO1 the $\text{tRNA}^{\text{Lys}}(1)$ gene close to *oprL*–*phnAB* is located between coding sequences PA0976 and PA0977. The 8.9 kb DNA block 3' of tRNA^{Lys} from PA0977 to PA0987 represents a non-conserved insertion that terminates with duplicated 22 bp of the 3' end of the $\text{tRNA}^{\text{Lys}}(1)$ gene, presumably the former attP-site of the integrated element. This 8.9 kb block of PAO1-specific DNA is absent in clone K strains, harboring PA0988 as their first PAO1 homolog downstream of $\text{tRNA}^{\text{Lys}}(1)$.³⁵

In strain C a 23.4 kb large gene island termed PAGI-4 is integrated at this $\text{tRNA}^{\text{Lys}}(1)$ site.³⁹ PAGI-4 substitutes PA0977 to PA0994 and consists of two blocks of non-PAO1 sequence that each are flanked by short stretches of PAO1-homologous sequence. The first block of 9.5 kb of non-PAO1 sequence flanked by truncated versions of PA0977 and PA0980, shares conserved synteny and 87–99% amino acid sequence with ORFs of PAGI-2, PAGI-3, and pKLC102 (see below). The second 12.7 kb DNA segment flanked by truncated versions of PA0981 and PA0994 encodes the typical elements of a transposon similar to Tn4652 from *Pseudomonas putida*.

Strain PA14 carries the 10.7 kb island PAPI-2 at this location that shares substantial sequence similarity with the PAO1 genome island.³⁰ The PAO1 pyocin genes PA0984-85 are replaced in PAPI-2 by the cytotoxin *exoU* gene and its chaperone *spcU*, and accordingly PAPI-2 has termed a pathogenicity island. In two clinical isolates another 81 kb island that also contains the *exoU* gene has been identified to again reside at the very same genomic position.⁹⁵ In summary, five different genome islands varying of 8.9–106 kb in size have yet been identified to integrate into the tRNA^{Lys}(1) gene close to *oprL-phnAB*.

Three different genome islands PAPI-1, pKLC106, and pKLC102 are known to insert into the tRNA^{Lys}(2) gene close to *pilA*. pKLC106 and pKLC102 are highly homologous plasmids. Clone K and clone C strains from the environment harbored chromosomal and episomal copies of this mobile genetic element.³⁹ PAPI-1, pKLC106, and pKLC102 share numerous features: approximate size (108, 106, 102 kb), a tRNA^{Asp}, tRNA^{Pro}, and tRNA^{Lys} gene cluster at their leftward PAO1 junction, and a direct repeat of the 3' half of the tRNA^{Lys} gene at their right border, and the integrase and the chromosome partitioning genes at the ends of the island, similar to PAPI-2 and PAPI-3.^{30,39} PAPI-1 is a pathogenicity island because it carries at least 19 virulence factors that occur on genomic islands found in a wide spectrum of other pathogenic bacteria.³⁰ pKLC102 contains the 8.5 kb *chvB* gene homologs of which are known to confer host tropism and virulence and to be essential for the interaction of the bacterium with its eukaryotic host. PAPI-1 and pKLC102 encode type IV group B pili and type IV thin sex pili, respectively, and share a set of homologs found as island-specific genes in PAPI-2, PAPI-3 (see above), and numerous genome islands in other proteobacteria (Figure 6). Fifteen of 33 core genes common to 15 genome islands from β - and γ -Proteobacteria were congruent with the phylogenetic relationships of each of the individual genes indicating that all five large genome islands known so far in *P. aeruginosa* belong to one family of related syntenic genomic islands with a deep evolutionary origin.⁵¹ The mobile pKLC102 shares with PAPI-1 the phage module that conferred integrase, the *att* element and the syntenic set of genes, but it differs from PAPI-1 in carrying a plasmid module that conferred *oriV* and genes for replication, partitioning, and conjugation.³⁹

The only large genome island known so far that is not associated with a tRNA gene is the 49 kbp PAPI-1. This first described genome island in *P. aeruginosa* is widely distributed in the population.⁴⁵ The island was probably assembled from two ancestral components of different G + C content. 35 kb of the higher G + C content portion is also found in the *P. putida* KT2440 genome.⁵³ PAPI-1 contains genes potentially involved in oxidative stress resistance, and replaced PAO1 genes PA2218 to PA2222. Furthermore, in other *P. aeruginosa* strains, this region contains an insertion of 3 kbp of DNA unrelated to PAPI-1.

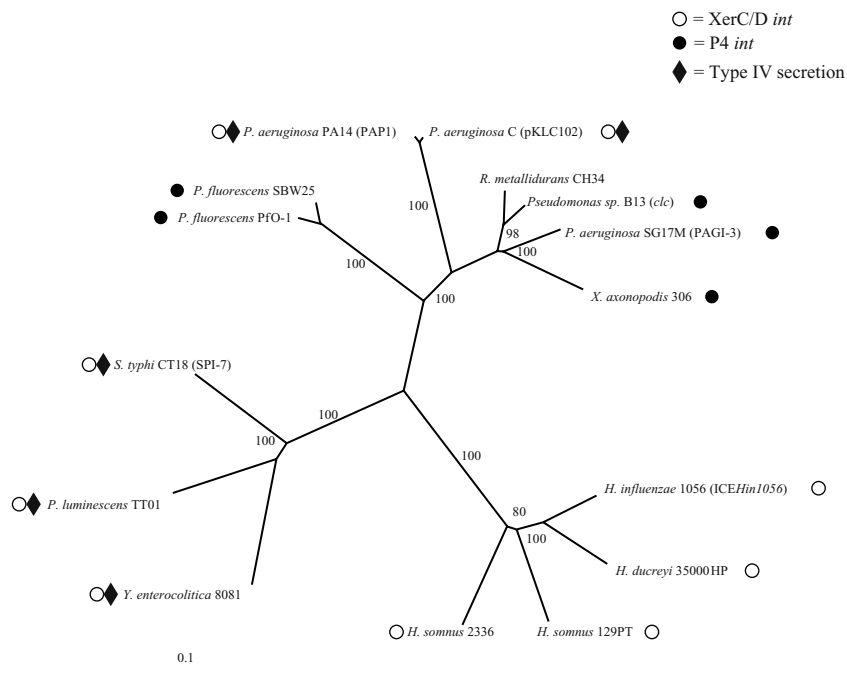


Figure 6. The amino acid sequences of the 15 predicted sequences common to 15 genome islands were concatenated and aligned by ClustalX. The alignment of each of the genes alone was consistent with the alignment illustrated. Reproduced from the article by Mohd-Zain *et al.*⁵¹ with permission by the authors and the American Society for Microbiology.

In summary, the accessory genome of *P. aeruginosa* is made up of numerous genome islets and islands. Most genome islands analyzed so far are integrated into tRNA genes and share a signature of syntenic genes that are widespread among proteobacteria. Accessory genes are nestled among these core genes and confer a diverse repertoire of strain-specific features.

2.3. Population Biology of *P. aeruginosa*

Bacteria can have population structures ranging from the fully sexual to the highly clonal. Several independent studies in the last years demonstrated that *P. aeruginosa* has a nonclonal population structure punctuated by highly successful epidemic clones or clonal complexes.^{14,36,52,60,76}

By applying multilocus SNP typing on two unrelated strain collections, the index of association was consistently calculated in two independent studies to be 0.29¹⁴ and 0.31³⁶ indicating that *P. aeruginosa* has a nonclonal population

structure. The index of association is a measure of the extent of linkage equilibrium within a population by quantifying the amount of recombination among a set of sequences and detecting associations between alleles at different loci. Comparisons of the topologies of neighbor-joining trees for the nucleotide sequences of individual loci revealed in both studies that there was little, if any, congruence between the trees. Strains, which belong to the same genotype, are characterized by non-random association of alleles that is not disrupted by recombination. In contrast, the recombination frequency of large chromosomal segments between genotypes is high enough to break up clonal associations and have all genotypes in linkage equilibrium to each other. Hence, the *P. aeruginosa* genotypes are equivalent biovars that form a net-like population structure. Each genotype represents a cluster of closely related strains (clonal variants) that share identical alleles.³⁶

When typing a strain, the core genome can be represented by the multilocus SNP genotype of conserved genes whereas both core and accessory genome can be represented by the PFGE-separated macrorestriction fragment profile. By comparative SNP and *SpeI* PFGE genotyping⁵² numerous cases were resolved whereby strains shared the SNP genotype but had different *SpeI* macrorestriction profiles. Interestingly, this finding applied to the most abundant *SpeI* genotypes. Further examples were the completely sequenced strains PAO1 and PA14 isolated in Australia and the US, respectively, which shared their SNP genotype with numerous clinical and environmental isolates from Europe. This data indicate the high proportion of dominant epidemic clones in the *P. aeruginosa* population. These epidemic clones such as the European clone C, the Australian, and the UK epidemic clones have unrelated genotypes, suggesting that they have evolved independently.^{14,76}

RFLP analysis of the chromosome and SNP analysis of individual genes measure different evolutionary forces. The conservation of the SNP genotypes and the divergence of *SpeI* macrorestriction patterns in strains sharing the same SNP profile agree with the idea that the core genome of *P. aeruginosa* is highly conserved and that its evolution and structure rely more on acquisition, loss, and rearrangements of genome islands and genome islets than on point mutations. In other words, horizontal gene transfer has a more important role than point mutations on the evolution of *P. aeruginosa* in most habitats. The only known exception seems to be the uncommon habitat of the human respiratory tract where a high proportion of hypermutable *P. aeruginosa* strains emerges over time.⁵⁶

In enterobacteria a single genotype predominates one habitat (Figure 7). Genotypes are associated with particular pathogenicity islands which result in disease-associated clones. In contrast, there is no correlation between *P. aeruginosa* clones and habitats (Figure 7). Dominant clones are ubiquitously distributed in both disease and environmental habitats: for example, members of the same clone were recovered from oil shale and from the lungs of patients

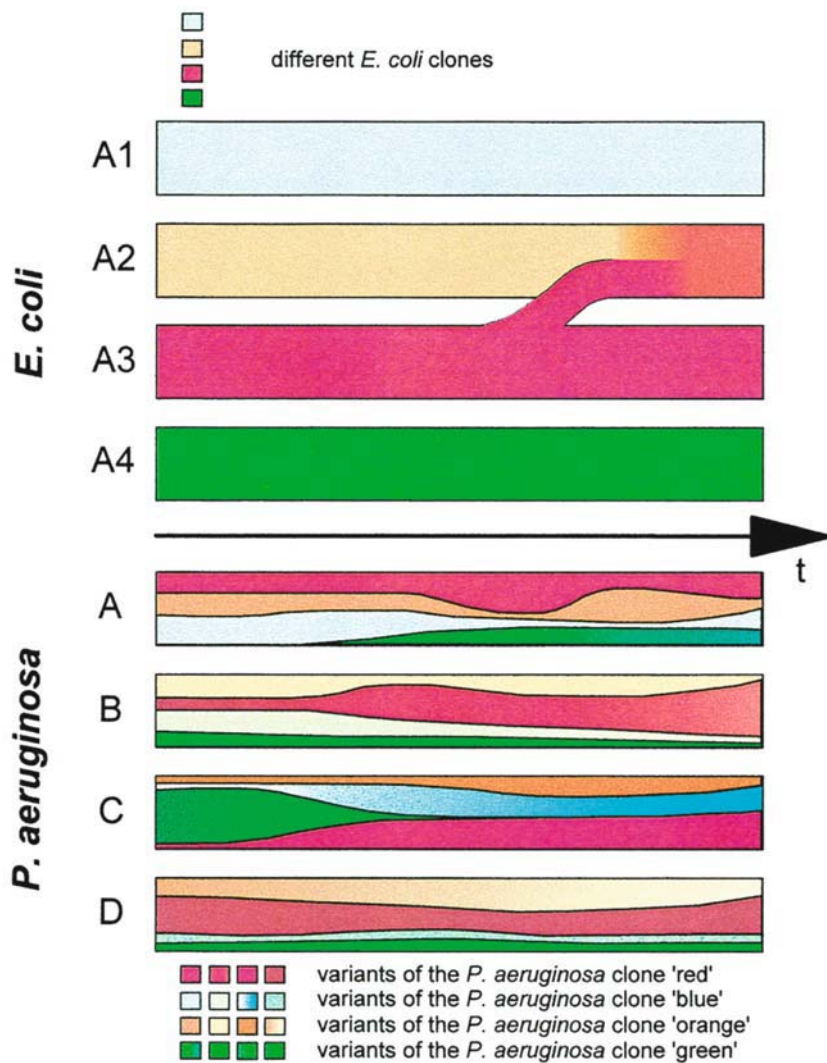


Figure 7. Clonal population structure of *E. coli* and *P. aeruginosa* differing in spatio-temporal distribution. The related disease habitats of *E. coli* are designated A1, A2, A3, and A4; the diverse disease and environmental habitats of *P. aeruginosa* are symbolized by A, B, C, and D. Whereas *E. coli* shows a clear correlation between clone and habitat (disease-associated clones), being only occasionally interrupted by horizontal gene transfer, the same spectrum of *P. aeruginosa* clones colonizes even unrelated habitats. Individual variants of a certain clone may predominate in several niches. Variants of *P. aeruginosa* clones undergo adaptive genetic changes, suggested by the shading. Reproduced from the article by Kiewitz and Tümmler³⁶ with permission by the authors and the American Society for Microbiology.

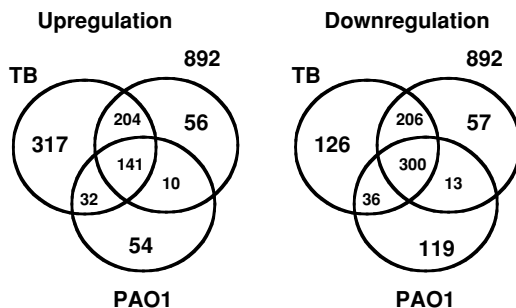


Figure 8. Transcriptome analyses in strain PAO1 and the two clone TB strains TB and 892: PAO1 genes with significantly changed expression levels in hydrogen peroxide treated *P. aeruginosa* TB, 892, and PAO1. Genes that were found to be up- or down-regulated in two or three strains are indicated in the intersections.

with CF.^{23,52} Disease and environmental isolates of *P. aeruginosa* clones are indistinguishable in their genotypic and chemotaxonomic properties^{14,23,52} and are functionally equivalent in several traits relevant for their virulence and environmental properties.¹ In summary, *P. aeruginosa* appears to be so versatile that it can colonize a variety of different ecological niches without specialization (Figure 7).

3. CLONAL VARIATIONS OF PHENOTYPE

The polymorphic loci in the *P. aeruginosa* core genome lead to a clone-specific repertoire of pyoverdines, LPS, pili, and flagella, whereby the latter two do not only vary in the primary amino acid sequence, but also in the posttranslational glycosylation pattern. Most interclonal variations are conferred by the genome islets and islands of the accessory genome, but their impact on phenotype has yet not been resolved. The differential sets of O-antigens, pyocins, and phage receptors have been exploited for decades for strain typing, but the first systematic comparisons of global mRNA transcript and protein profiles in genetically typed strains have only reported recently.

Salunkhe *et al.*^{69,70} compared the inter- and intraclonal diversity of the PAO1 transcriptome between strain PAO1 and the two clone TB strains TB and 892 under different environmental conditions. The number of expressed genes differed by clone. The PAO1 strain expressed 64% of its genes in LB medium, whereas the TB and 892 strains expressed more than 70% of genes. When the strains were exposed to the stressor hydrogen peroxide, 24, 17, 12% of the 5900 ORFs were significantly differentially regulated in *P. aeruginosa* TB, 892 and PAO1, respectively. Only 441 genes were consistently differentially expressed in the three strains. 729 genes showed strain-specific responses and 501 genes

were similarly regulated in two strains (Figure 8). The expression profile was significantly more related between the two members of the TB clone than between either strain with PAO1. The 892 strain shared 84% and 88% of up- and down-regulated genes with its more virulent clonal variant TB that exhibited a more strain-specific expression profile with 50% and 76% of up- and down-regulated genes being in common with 892. It is noteworthy that strains TB and 892 exhibit identical SNP and *SpeI* genotypes⁵² and that the DNA sequence was 100% identical in more than 100 kb of randomly selected loci of the core genome.³⁶ Nevertheless the global mRNA expression profiles of the two clonal variants were divergent when the strains were cultivated simultaneously in LB medium in the presence or absence of oxidative stress. Apparently there are a few sequence variations in some key genes that account for the divergent phenotypes. In other words, genetically very closely related *P. aeruginosa* strains present a strain-specific mRNA expression profile that under carefully controlled identical conditions is highly reproducible, but distinct from members of the same clone.

The proteome of the two clonal variants TB and 892 has also been compared by two-dimensional polyacrylamide gel electrophoresis coupled to mass spectrometry to map the extracellular, intracellular, and surface sub-proteomes and to identify differentially expressed proteins.³ About 4% of all detected protein spots were differentially expressed between both strains including absent or present spots and spots with a more than two-fold changed intensity. Nineteen of 78 differentially expressed spots were identified by mass spectrometry on the basis of a predicted gene product in the genome database for *P. aeruginosa* PAO1, for 13 additional spots mass fingerprints were obtained which most likely represent clone-specific proteins of the TB lineage. Many of the protein spots in TB that were missing or expressed at lower levels in the less virulent 892 strain were identified as quorum-sensing regulated virulence factors.

Strains of *P. aeruginosa* can be phenotypically classified by their mode of pathogenicity as either invasive, where the bacterium is internalized by host cells, or cytotoxic, where the host cell is killed without internalization through the expression of cytotoxicity factors. These phenotypes are thought to depend primarily on the interactions of pseudomonal membrane and secreted proteins with host cells. Nouwens *et al.*⁵⁴ compared the proteomes of the outer membrane and extracellular protein-enriched fractions from the invasive strain PAO1 and the cytotoxic strains 6206. Membrane protein strain differences were typically the result of minor amino acid sequence variations resulting in small mass and isoelectric point shifts visible on two-dimensional gels. Analysis of extracellular proteins from stationary phase growth, however, revealed significantly different protein profiles between the two unrelated clones. Extracellular fractions from the invasive PAO1 strain were dominated by extracellular proteases including elastase (LasB), LasA protease, and chitin-binding protein, as well as several previously designated 'conserved hypotheticals' of unknown function. Conversely, extracellular fractions from strain 6206 consisted mainly of

cellular and membrane exposed proteins including GroEL, DnaK, and flagellar subunits. These are thought to result from cellular turnover during growth and the reliance on the secretory mechanisms of this strain to produce high levels of cytotoxicity factors, such as ExoU, which may be produced only upon specific interactions with host cells.

Wehmhöner *et al.*⁹² compared the proteome profiles of six *P. aeruginosa* clones grown in modified minimal Vogel-Bonner medium. The proteome analysis revealed almost identical patterns for the cellular extracts, whereas interclonal diversity were demonstrated for the secretomes of cultured *P. aeruginosa*. The diversity was even greater for the immunogenic protein patterns expressed *in vivo*. The observed interclonal variability of the secretome may reflect the differential, clone-specific regulation of gene expression and/or the utilization of genes that are not encoded by the core genome but are encoded by the highly dynamic accessory genome. To differentiate between the two mechanisms, Wehmhöner *et al.*⁹² also analyzed the proteomes of sequential clonal variants with diverse morphotypes. A *P. aeruginosa* isolate that formed irregularly shaped colonies was compared with a hyperpilated and autoaggregative *P. aeruginosa* small colony variant. The expression profiles of cellular extracts of the two morphotypes exhibited only minor differences, in contrast to the marked differences in the expression profiles of the extracellular fractions. Mass spectrometry revealed that the small colony variant overexpressed proteins secreted by the type I and type III secretion systems. This finding implies that the variability of the secretome is due to differential regulation of protein expression, possibly as a consequence of small adaptational mutations. These observations were backed up by genome-wide transcriptional profiles of the two clonal morphotypes.⁹¹ Of the more than 300 differentially expressed genes, the upregulation of the type III secretion system and the respective effector proteins in the small colony variant was the most striking finding. The conserved intracellular proteome of strains grown *in vitro* probably reflects the fact that the need for adaptation under these conditions is low, and inter- and intraclonal differences that reflect the versatility of niche specialists are not likely to be detected. Moreover, the cellular proteome comprises mostly proteinaceous cell constituents that are expected to be species-specific but not clone- or strain-specific. However, the secretome expression is strongly strain and morphotype-specific. Since the secreted *P. aeruginosa* proteins come into direct contact with their environment, they could be especially important and thus be essential for bacterial adaptation. Moreover, the secretome includes important virulence factors essential for establishment of an infection within the human host. In summary, the secretome is a sensitive measure of *P. aeruginosa* strain variation.

A special case of intraclonal diversity are the strain variations that occur during subculturing *in vitro*. A timely and important example is the completely sequenced reference strain PAO1. The sequenced strain⁸⁵ differs from the ancestor strain that had been independently physically mapped in Australia and Germany,³³ by a 1.7 Mbp inversion between the *rrnA* and *rrnB* loci and an about

20 kbp deletion close to the *rrnC* locus. Moreover, PAO1 stocks maintained at different laboratories are not identical in phenotypic traits, a spectacular example being the differential virulence in infection models even though the strain had been originally obtained from the same public collection (own unpublished data). During storage and subculturing the PAO1-derived isolates apparently diversified by inversion, deletion, and point mutation.

4. INTRACLONAL EVOLUTION AND DIVERSITY IN CLINICAL HABITATS

4.1. Hospital-Acquired Infections

P. aeruginosa is resistant to many antimicrobial agents and a major source for nosocomial infections in predisposed individuals. Hence, the major practical issue to assess clonality and intraclonal evolution are infection control measures to determine epidemic clonality amongst multidrug-resistant strains or to document outbreaks of (drug-resistant) clones (as examples see refs [34,50,58,59]). A few groups combined the molecular epidemiology of hospital-acquired infection with the characterization of intraclonal diversity.

Hocquet *et al.*³² retrospectively analyzed the intraclonal variation of drug resistance of a serotype O:6 multidrug-resistant *P. aeruginosa* clone during a 4-year long outbreak at a French University Hospital. This clone was initially recognized because of its particular susceptibility profile to aminoglycosides [conferred by an ANT (2'')-I enzyme] and fluoroquinolones (caused by mutations in the QRDR of *gyrA* and *parC*) and because of its elevated resistance to many β -lactams. The susceptibility profile of this epidemic clone to fluoroquinolones and aminoglycosides was relatively stable during the outbreak but showed important isolate-to-isolate variations in the susceptibility to β -lactams. Analysis of 18 genotypically related isolates selected on a quarterly basis demonstrated alterations in DNA topoisomerases, constitutive overexpression of the MexXY efflux system, derepression of intrinsic AmpC β -lactamase and sporadic deficiency in the carbapenem-selective porin OprD. Of the 18 isolates, 14 were also found to overproduce the efflux system MexAB-OprM as a result of alteration of the repressor protein MexR. Of the four isolates exhibiting wild-type MexAB-OprM expression despite the MexR alteration, two appeared to harbor secondary mutations in the *mexA-mexR* intergenic region and one harbored secondary mutations in the putative ribosome binding site located upstream of the *mexAB-oprM* operon. In conclusion, many mechanisms were involved in the multiresistance phenotype and the clone sporadically underwent substantial genetic and phenotypic variations during the course of the outbreak.

P. aeruginosa is responsible for severe nosocomial pneumonia in mechanically ventilated patients. Denervaud *et al.*¹⁶ collected 442 *P. aeruginosa* isolates during the first 3 days of documented colonization of 13 intubated

patients in order to study quorum-sensing dependent phenotypic traits. The 442 isolates belonged to nine different clones. Eighty-one percent of the isolates produced homoserine lactones and quorum-sensing dependent extracellular virulence factors, including total exoprotease, elastase, HCN, pyocyanin, and rhamnolipids, at levels equivalent to those of the reference strain PAO1, but 19% of the isolates were deficient in cell-to-cell signaling, because the *lasR* gene encoding the LasR transcriptional regulator was inactivated by various mutations. A subset of these isolates also had mutations in the *rhlR* gene, probably explaining the defect in both homoserine lactone and extracellular virulence factor production. Since the homoserine lactone production of these strains was complemented by the chromosomal insertion of the wild-type *lasR* and *rhlR* genes, additional mutations are unlikely. Three of the 13 patients presented a *P. aeruginosa* pneumonia as a complication of their respiratory colonization of whom two subsequently developed a *P. aeruginosa* bacteremia. These bacteremic isolates were clonal variants carrying the *lasR* or *lasR/rhlR* mutants. This is the first report on clinical isolates that are unable to produce cell-to-cell signals as a result of both *lasR* and *rhlR* mutations, and it is interesting to note that the intraclonal evolution toward loss of quorum-sensing was associated with the gain-of-invasiveness to breach the airway epithelial barrier.

4.2. Cystic Fibrosis

Most information about the evolution of intraclonal diversity of *P. aeruginosa* was obtained from retrospective cross-sectional and longitudinal analyses of isolates recovered from the atypical habitat of the CF lung. CF is a severe monogenic disorder of ion transport in exocrine glands that is caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene. The basic defect predisposes to chronic bacterial airway infections, particularly with *P. aeruginosa*. The *P. aeruginosa* infections in CF are a paradigm of how environmental bacteria can conquer, adapt, and persist in an atypical habitat and successfully evade defense mechanisms and chemotherapy in a susceptible host. Airway infections with *P. aeruginosa* in individuals with CF are unique in that they chronically affect a host who is immunocompetent in terms of cellular and humoral responses but is immunocompromised by impaired airway clearance. Once *P. aeruginosa* has taken residence in the CF lungs, the organism is notoriously resistant to eradication by chemotherapy. The pseudomonads chronically colonize the bronchiolar lumen and virtually never breach the epithelial barrier (reviews: refs [26,89]).

4.2.1. Clonal Variations of Genotype in CF Lungs

Most individuals with CF become chronically colonized with a single clone of *P. aeruginosa* that stays in the lungs for many years. Turnover of clones was seen in the author's laboratory after 5–15 years in about half of the

investigated patients. Transient or permanent co-colonization with more than one clone was seen in 20–30% of patients. These conditions that CF lungs are chronically infected for years by one or a few *P. aeruginosa* clones are precisely those that theoretical studies predict for the evolution of mechanisms that augment the rate of variation. Determination of spontaneous mutation rates in 128 *P. aeruginosa* isolates from 30 CF patients revealed a high proportion (20%) with an increased mutation frequency (mutators).^{55,56} Seven out of 11 analyzed CF mutator strains were found to be defective in the mismatch repair system. The alterations in the *mutS*, *mutL*, and *uvrD* genes were found to be responsible for the mutator phenotype. In four cases (three *mutS* and one *mutL*), the genes contained frameshift mutations. The fourth *mutS* strain showed a 3.3 kb insertion after the 10th nucleotide of the *mutS* gene, and a 54 nucleotide deletion between two eight nucleotide direct repeats. This deletion, involving domain II of MutS, was found to be the main one responsible for *mutS* inactivation. The second *mutL* strain presented a K310M mutation, equivalent to K307 in *E. coli* MutL, a residue known to be essential for its ATPase activity. Finally, the *uvrD* strain had three amino acid substitutions within the conserved ATP binding site of the deduced UvrD polypeptide, showing defective mismatch repair activity. In summary, intraclonal evolution of *P. aeruginosa* in CF lungs can be driven by hypermutable clonal variants. Since the proportion of mutators in the population increases over time, point mutations preferentially accumulate during the late stages of the infection.

Mutator strains were not found in 75 non-CF patients acutely infected with *P. aeruginosa*,⁵⁶ but were also seen in 30 patients with non-CF underlying chronic respiratory diseases (22 with bronchiectasis and 8 with chronic obstructive pulmonary disease).⁴⁶ Seventeen of the 30 patients were colonized with hypermutable strains. The *mutS* gene was inactivated in isolates from 11 patients. Multiple antimicrobial resistance was documented in 42% of the hypermutable strains in contrast to 0% of the non-hypermutable strains. This study demonstrates that first, hypermutation is a key factor for the emergence of the multidrug resistance phenotype; and that second, in contrast to what has been described in acute processes, hypermutable *P. aeruginosa* strains are highly prevalent in chronic infections of the human respiratory tract.^{46,56}

Besides the mismatch repair system and the targets that confer multiple antimicrobial resistance, further known hotspots for mutation in CF isolates are the *mucA* and *lasR* genes. Inactivation of *lasR* is often causative for the loss of production of *N*-acylhomoserine lactones (AHL) which is not rare in CF, particularly if the lung is co-colonized with *Burkholderia cepacia*.^{19,24} The mucoid phenotype of *P. aeruginosa* has been linked to mutations in a gene cluster designated as the *mucABCD* genes that encode proteins that inhibit the activity of the alternative σ -factor AlgU.⁴⁹ When alginate production is minimal, AlgU (also referred to as AlgT) is bound in a complex along with MucA and MucB, but under environmental stress conditions this complex is disrupted,

leading to the release of AlgU into the cytosol. AlgU acts on the key alginate biosynthesis gene *algD*, which encodes a GDP mannose dehydrogenase, and on *algR*, a response regulator genes that increases alginate synthesis.⁷⁴ Mutations in *mucA*, *B*, and *D* are held responsible for alginate overproduction and conversion to a stable mucoid phenotype in *P. aeruginosa*, while mutations in *mucC* do not cause any overt effects on alginate synthesis.^{9,49} Consistent with this hypothesis, mutations in *mucA* have been detected in mucoid *P. aeruginosa* strains isolated from chronically infected CF patients.^{2,9,10,75,84} Alginate production is also dependent on a second alternative σ -factor, RpoN, and likely on other mutations in genes which are not known at present. However, according to more recent studies mutations in the *mucABD* cluster are not exclusively correlated to overexpression of alginate in *P. aeruginosa* CF isolates.^{11,84} The combined analysis of quantitative alginate expression and *mucA*, *mucB*, *mucD*, and *algU* sequencing in 37 *P. aeruginosa* strains revealed that a distinct proportion of phenotypically non-mucoid *P. aeruginosa* strains carried *mucA* stop mutations which were also present in alginate-overexpressing, mucoid *P. aeruginosa* strains.¹¹ Since sequence analysis of *algU* did not reveal any mutational genetic changes, other, unknown, mechanisms are presumably regulating alginate expression in these *mucA* mutated strains.

P. aeruginosa in CF lungs is not only prone to point mutations, but also to gross changes of the chromosomal frame. When Ernst *et al.*²⁰ analyzed 13 isolates from seven young CF children by hybridization on PAO1 whole genome DNA microarrays, they detected 2 strains with large deletions (strain CF250: 119 kb (PA1909–PA2010); strain CF5296: 189 kbp (PA2273–PA2409). The latter deletion eliminates the hypervariable pyoverdine locus.

Reversible genome rearrangements were seen in CF isolates which were carrying the mobile genetic element pKLC106. pKLC106 reversibly recombined with sequential clone K chromosomes at one of the two tRNA^{Lys} genes³⁵ (Figure 9). In all investigated sequential clone K CF strains both episomal and chromosomal copies were detected.

Physical mapping of 18 CF clone C isolates revealed inversions in eight strains, two of which were harboring two nested inversions.⁶⁷ Besides one small scale inversion of 40 kb, the inversions ranged from 1 to 5 Mbp whereby their recombination endpoints scattered on the chromosome. In six cases the region of the terminus of replication was included in the recombinational exchange and was shifted by maximal 17% of genome size (Figure 4). A hotspot of recombination was mapped to the pKLC102 locus.⁴⁰ All investigated clone C isolates from aquatic habitats and the hospital environment harbored chromosomal and episomal copies of pKLC102. However, many isolates from CF lungs contained either no (C5) or only chromosomally integrated pKLC102 (C2) (Figure 9). Of the four subgroups of clone C,⁶⁷ subgroup C was exclusively represented by CF lung isolates and differed from the other three groups by the insertion of the class 1 composite transposon TNCP23 into chromosomally

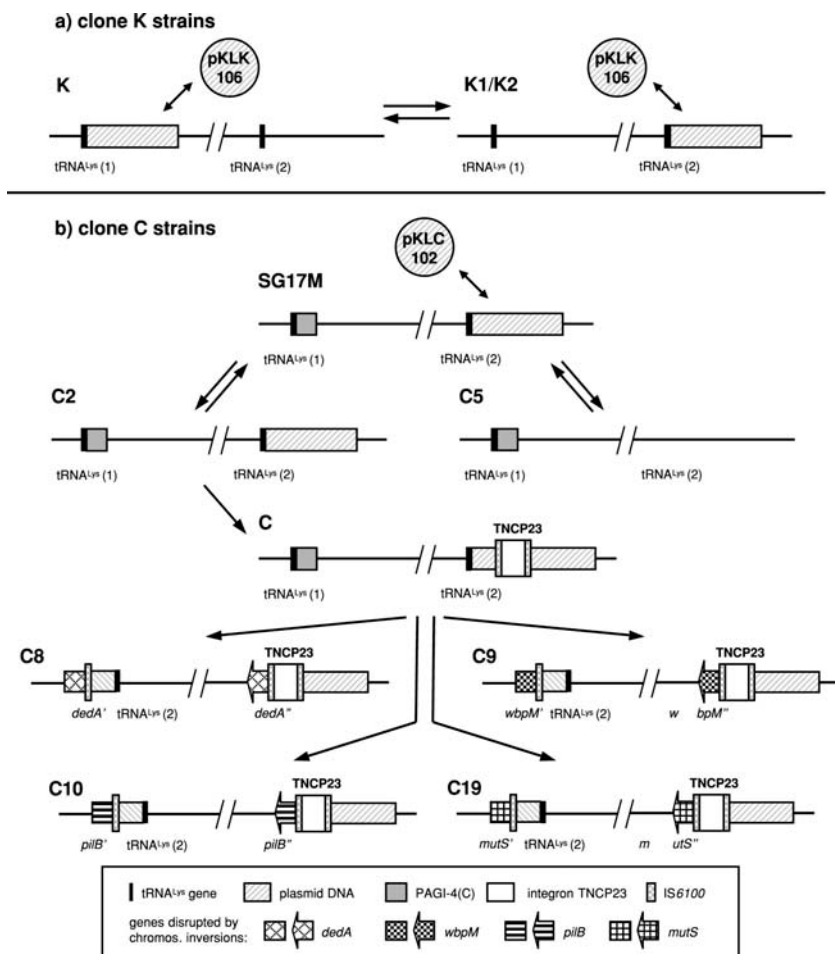


Figure 9. Evolution of *P. aeruginosa* strains linked to plasmid DNA. (a) Reversible integration of plasmid DNA into two possible sites of clone K strains. (b) Different forms of plasmid DNA in clone C strains. In subgroup SG17M pKLC102 is found episomally and integrated into the genome at tRNA^{Lys}(2). Strain C5 apparently lost the pKLC102 DNA, while strain C2 only harbors the integrated form. In subgroup C the integron TNCP23 inserted into chromosomally integrated pKLC102. Free plasmid is not detectable in subgroup C strains indicating that TNCP23 prevented mobilization. TNCP23 is flanked by copies of IS6100. Intramolecular transposition of the left copy of IS6100-L is coupled with an inversion of the chromosomal region between the transposed copy and IS6100-L in some strains of subgroup C. For these strains C8, C9, C10, and C19, the tRNA^{Lys}(1) area is not shown. Reproduced from the article by Klockgether *et al.*³⁹ by permission of the authors and the American Society for Microbiology.

integrated pKLC102, which may have been acquired because of the encoded *aadB* gene conferring gentamicin resistance (Figure 9). *P. aeruginosa* converges in CF lungs to a common phenotype characterized by the decreased production of membrane components, cellular appendages and secreted factors (see below). This phenotypic signature was partially gained in subgroup C strains by TNCP23-mediated chromosome remodeling. Intramolecular transposition of the active IS-6100 element of TNCP23 led to large chromosomal inversions which disrupted genes that are typically inactivated during the adaptation of *P. aeruginosa* to the atypical habitat of CF lungs (Figure 9). In parallel the integrity of pKLC102 was destroyed. The two attachment sites were separated so that the genetic contents of pKLC102 was irreversibly fixed in the chromosome. In summary, Figure 9 visualizes the evolution of plasmid pKLC102 from a mobile genetic element to an irreversibly fixed genome island that finally was disrupted and distributed to separate chromosomal regions. It should be noted that the increasing complexity of genome organization caused by insertion, transposition, and inversion was accompanied by mutation, deletion, and/or duplication of sequence close to the breakpoint.

In summary, inversions, deletions, and point mutations are common for the intracolon evolution of *P. aeruginosa* in CF lungs.

4.2.2. Clonal Variations of Phenotype in CF Lungs

Apart from the evolution of genome organization, *P. aeruginosa* develops common phenotypic features irrespective of clonal descent. This phenotypic conversion is characteristic for the CF lung habitat and, on the whole, is genetically fixed and irreversible. In other words, some aspects of intracolon evolution are similar in all clones. Strains become LPS deficient (non-typable or polyagglutinating rough strains) and sensitive to lysis by complement.²⁷ On the other hand, CF isolates produce modified lipid A forms containing palmitate and aminoarabinose that are associated with resistance to cationic antimicrobial peptides and stronger induction of inflammatory responses such as interleukin 8 expression.²¹ *P. aeruginosa* strains vary in their differential repertoire of bacteriophage receptors and the production of pyocins, which lyse susceptible *P. aeruginosa* strains. These differential properties are gradually lost in most *P. aeruginosa* during chronic colonization of the CF lung. Susceptibility to phages and secretion of pyocins decrease within a few years time.⁶⁵ The production of the major siderophore pyoverdine also changes over time. Pyoverdine-negative mutants emerge, but retain the capacity to take up pyoverdines.¹⁷ CF strains become immotile owing to the loss of their flagella.⁴⁷ CF isolates from early colonization were highly motile and expressed both flagellin and pilin. However, about 40% of more than a 1000 examined isolates from chronically colonized CF patients lacked flagellin expression and were nonmotile. Sequential isolates remained consistently nonmotile. Lack of motility was rare among environmental isolates (1.4%) and other clinical isolates (3.7%) of *P. aeruginosa* examined.⁴⁷

Moreover, during chronic colonization of the CF lung most *P. aeruginosa* strains reduce or even abolish the production of type II and III secretion effector proteins and thus reduce cytotoxicity. While the killing of epithelial and phagocytic cells may be an important feature of acute infections, the same virulence mechanisms appear to be incompatible with chronic colonization of CF patients.²⁶ *P. aeruginosa* isolates were examined that had been obtained from 7 patients soon after their initial colonization and then again more than a decade later, after the establishment of chronic lung infections.⁴⁴ Early isolates were typically cytotoxic, the exception being the highly mucoid strains. Variants of the same clone, isolated years later from the same patients, were found to be nontoxic, suggesting that there has been a selection for loss of cytotoxicity. In many cases restoration of type III regulation, through the expression of the ExsA regulator, was able to reestablish ExoS secretion and cytotoxicity. However, this was not the only mechanism of attenuation since expression of ExsA was not able to restore ExoS secretion in all of the clinical isolates. Moreover, some strains accumulated more than one mutation in the type III secretion system so that ExoS secretion could be restored with ExsA expression; but cytotoxicity was still attenuated. What was also apparent from the pairwise comparisons of early and late isolates was that the phenomenon of delayed cytotoxicity associated with type II secretion effector proteins was also lost in later isolates. The respiratory tracts of CF patients therefore provide a strongly selective environment for the accumulation of pathoadaptive mutations, which favor a chronic existence that often necessitates the elimination of a potent cytotoxic mechanism.

Another common feature of intraclonal evolution in the CF lung is the diversification of morphotype, the hallmarks being the emergence of small colony variants^{28,29} and mucoid colonies.^{26,57} The alginate-overexpressing mucoid phenotype is typical for CF isolates and very uncommon in other habitats.²⁶ A subgroup of hyperpilated small colony variants is prone to biofilm formation and induction of type II and type III secretion^{29,91} which leads to increased virulence in infection models in contrast to the notion that most *P. aeruginosa* isolates from chronically colonized CF lungs are typically attenuated in virulence (see above). Auxotrophy is common in CF, particularly in those with severe underlying pulmonary disease.⁸⁷ At this late stage the auxotroph count exceeds more than 50% total CFU. The majority of auxotrophs required methionine as the sole factor.⁸⁶ In summary, the common conversion of phenotype of *P. aeruginosa* in CF lungs starts with morphotype diversification and loss of outer membrane constituents and cell appendages and ends with a progressive change from proto- to auxotrophy. At this final stage of adaptation to the CF lung, the accumulation of pathoadaptive mutations will probably impair the fitness of the bacteria to grow in other habitats.

Besides this convergence in phenotype that progressively happens over time, numerous phenotypic features are fluctuating in the *P. aeruginosa* CF

lung communities. These periodic changes in phenotype probably result from the emergence and disappearance of clonal variants with differential fitness. The repeated courses of regular antipseudomonal intravenous chemotherapy and the long-term administration of aerosolized aminoglycosides inadvertently will select for resistant variants, and correspondingly fluctuations in the susceptibility patterns to antipseudomonal agents are a common finding in CF sequential isolates.

The adhesion phenotypes also vary strongly over time. *P. aeruginosa* mainly resides in the bronchiolar lumen of the CF airways embedded into a matrix of DNA, bacterial exopolysaccharides and human mucins.⁸⁸ *P. aeruginosa* uses chiefly proteins of its flagellar apparatus to initiate this binding and recognizes a variety of oligosaccharides that have been identified in mucins.^{6,62} Among these are both neutral oligosaccharides and several forms of acidic oligosaccharides derived from the Lewis antigens.⁷² Serial *P. aeruginosa* isolates from CF patients with advanced lung disease were characterized in their binding to CF human tracheobronchial mucins from three of these patients.⁸⁸ The strains differed strongly in their specificity for and affinity to mucin carbohydrate. Intra- and interclonal variation was equally pronounced indicating that the mucin-binding phenotype is not conserved within a particular clone.

Binding capacity to the airway epithelium is another trait subject to intraclonal variation. *P. aeruginosa* binding capacity to respiratory epithelial cells was studied in a representative panel of 634 sequential *P. aeruginosa* strains isolated from 26 CF patients, from the onset of colonization for up to 15 years of infection.⁴¹ Adherence was strongly varying between clonal variants sampled at the same or different times, albeit three types divergent in the temporal evolution were noted: predominantly high binders, predominantly low binders at all times, or a shift from high binders at early colonization to low binders later on. Patients chronically harboring high binders had a worse prognosis than the others indicating that adhesion to the airway epithelium is a relevant pathogenic trait for *P. aeruginosa* to colonize and to persist in the CF lung.

Intraclonal variation may also be caused by AHL-dependent signaling between *B. cepacia* and *P. aeruginosa*.²⁴ When patients became transiently co-infected with an AHL-producing *B. cepacia* strain, AHL production by the co-residing *P. aeruginosa* isolates was switched off. However, months after the last *B. cepacia*-positive sputum the initial *P. aeruginosa* AHL profile was regained suggesting that AHL-mediated cross-talk between the two pathogens may affect the virulence of the mixed consortium which, in turn, may have selected for *P. aeruginosa* mutants producing lowered amounts of AHLs.

In summary, the CF lung habitat triggers a conversion of bacterial phenotype, but *P. aeruginosa* retains enough flexibility to recognize its environment of host cells and polymers and to respond to selective pressures such as antimicrobial chemotherapy or co-colonization with other taxa.

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